A METHOD FOR STUDYING THE PERMEABILITY OF THE BLOOD VESSELS OF THE DENTAL PULP DURING ACUTE INFLAMMATION

By

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INTRODUCTION

Until recently, investigations of inflammation of the pulp have used classical histo-pathological techniques. These studies, while they permit estimates of the damage associated with different operative procedures and dental materials, do not shed any great light on the process itself. Only a few studies of the dental pulp have treated inflammation as a continuous process and studied it in the living tissue.[1, 2, 3] In addition, most attention has been given to the cellular changes taking place such as leukocytic infiltration, while only rarely[4] are studies of the vascular changes made, changes which pathologists consider an important part of the defense mechanism.[5, 6, 7]

It is well known that an important portion of the inflammatory defense mechanism is humoral in nature. The vascular changes result in an accumulation of exudate in an inflamed area and this exudate is thought to dulute toxins and expose them to any natural antibodies present. The exudate can also coat the invader with a film of protein, making it more palatable to the leukocytes, and may limit the movement of organisms due to a barrier of coagulated fibrin.[5]

The hypothesis for the present study was that there is a difference in the amount of exudate formed when there is inflammation of the pulp compared to the amount formed when there is inflammation in the skin.

Recently there has been a renewed interest in the vascular changes that take place in inflammation.[8] The present

study is an attempt to adapt a method used for the study of the inflammatory process in the skin, to an investigation of the process in the dental pulp. It is hoped that the study might bring to light any differences between the skin and the dental pulp.

REVIEW OF THE LITERATURE

A brief summary of the current concept of inflammation will be presented with reference to the most relevant papers. This will be followed by a review of ideas concerning the fine structure of the smaller blood vessels both in the skin and in the dental pulp. The rest of the review will be concerned with the permeability of the vessels of normal and inflamed tissue.

Inflammation

The inflammatory response is described in the standard textbooks.[5, 6, 9] The reader is also referred to the recent review by Spector and Willoughby[7] and two new books.[10, 11] Theinflammatory process is a series of events rather than one single event and follows a similar course regardless of the noxious agent involved. Varying types of injury do however lead to variations in the intensity and duration of different phases of the reaction.

The events of inflammation fall naturally into two broad divisions — the response of the vessels and the cellular response.[7] The classic description of inflammation was made nearly a century ago by Cohnheim[12] who had brought together a number of observations by previous workers and then made many experiments of his own. His description of events as he saw them in the mesentry and tongue of the frog has not since been bettered. Cohnheim

observed that there is initially a dilatation of the injured vessels which occurs chiefly in the arteries, less in the veins, and least of all in the capillaries. With the dilatation which develops gradually, but which during the space of fifteen to twenty minutes usually attains considerable poroportions, there immediately sets in an acceleration of the blood stream, most striking again in the arteries, but very apparent in the veins and capillaries also. Yet this acceleration never lasts long; after half an hour or an hour it invariably gives place to a marked retardation in the velocity of the stream, the speed falling below normal.

Cohnheim[12] noted that in the affected veins there gradually developed a central column of red blood cells, flowing on in an uninterrupted stream of uniform velocity, surrounded by a peripheral layer of colorless cells. Later came the second characteristic division of the inflammatory reaction, the cellular response. The colorless blood corpuscles pushed out of the vessels and into the surrounding tissue.

While easy to describe, the concept of inflammation is difficult to define. Spector and Willoughby[7] defined it as the local reaction to injury of the living microcirculation and its associated tissues, in which would be included blood leukocytes and such features of perivascular tissue as mast cells and histiocytes. Payling Wright[5] includes the concept of protection, referring to inflammation as the process by which cells and exudate accumulate in irritated tissue and usually tends to protect them from further injury.

It was John Hunter[13] who first pointed out this protective

function of the inflammatory response which had previously been regarded as a distinct disease. Hunter showed that there was a difference between the causes of inflammation and the tissue reactions which follow those causes : "Therefore, inflammation is not to be considered as a disease, but as a salutary operation, consequent either to some violence or some disease." However, Hunter realized that the effects of inflammation were not always 'salutary'. The inflammatory reaction is a protective mechanism which does not always work to the advantage of the organism. For instance, it seems to serve no useful purpose in rheumatic and allergic diseases.

Most pathologists still regard the process of inflammation as a protective reaction, but Miles[14,15] has recently produced evidence that the tissues themselves play the major role in killing pathogens and that inflammation may be an unnecessary nuisance. We still do not know, in fact, what kills the invader in the early stages of infection nor precisely how microbial injury causes inflammation.

Inflammation in the dental pulp

By all accounts the response of the pulp to injury is essentially similar to that of other tissues.[16,17,18] The vascular changes are difficult to observe due to the enclosed nature of the pulp. Pohto and Scheinin[1] have been able to overcome this obstacle and their observations are the only ones we have to compare with those of Cohnheim.[12] Their

study showed that the process of inflammation in the incisor of the rat followed the classic pattern. There is yet to be a study published showing the process of inflammation in teeth with a closed apex.

One aspect of the vascular response of the pulp is that proposed by several authors[18, 19, 20] who have stated that the increase in the flow of blood results in such an increase in intrapulpal pressure that the walls of the arterioles and venules at the apex of the tooth are pressed closer and closer together. This constriction of the blood vessels may cause such a reduction in the flow of blood to the pulp tissue that there results necrosis and loss of vitality -- self-strangulation of the pulp. No confirmatory evidence has been presented for this view of events and recently Beveridge and Brown[2] have shown that pressure is not easily transmitted from one part of the pulp to another.

The fine structure of the smaller blood vessels

The classic concept of the structure of the smaller blood vessels, obtained from studies using the light microscope was given by Chambers and Zweifach,[21] in 1947. They considered that there was good evidence for the existence of three structural components of the capillary wall.

The first layer was the endothelium itself, consisting of pavement like cells separated by substantial amounts of intercellular cement substance. They thought that there was an endocapillary layer of absorbed plasma protein, although they had no direct microscopic evidence for this. The third

layer was the pericapillary sheath of argyrophil connective tissue fibers which served as an outer supporting layer.

Palade,[22] in 1953, was the first to publish an account of the fine structure of capillaries as seen with the electron microscope; however, since that time many more reports have been published on this topic.[23-30] There are considerable differences in the fine structure of capillaries depending on the tissues of which they are part. The description given here is true for dermal and muscle capillaries.

The intercellular junctions, as seen with the electron microscope, were not at all like the layer of 'cement' proposed by Chambers and Zweifach.[21] They were about 20 times too narrow to be seen with the light microscope.[30] The interpretation proposed was that the two cells were separated by a gap 100 to 150 Å in width,[25, 28, 29, 30] the cell membranes appearing dark in the area of closest attachment. With very high resolution a third dark line could be seen in some sections, in the middle of the gap. This line was thoght to represent a fusion of the outer layers of the cell membranes.[29, 30]

The endothelial layer was 0.1 to 0.3 micra thick except in the region of the nucleus where it was two to three micra thick.[30] The cells contained the normal complement of intracellular organelles but especially obvious was a system of spherical vesicles which were found in many other cells but with less frequency. These vesicles were uniform in diameter (600 - 700 Å) and occupied up to one third of the cell volume.[23-30] The deeper ones lay free while others made contact with the plasma membrane at the surface of the cell. Fernando and Movat[27]

observed small stomata with a diameter of 220 - 250 Å where the vesicles joined the cell membrane.

The majority of studies have failed to demonstrate an endocapillary layer.[23-28] When a red blood cell is pressed against the endothelium the two cells remain separated by an electron-translucent gap as narrow as 100 Å. Recently Luft[29] has shown that, when tissue was fixed in the presence of ruthenium red, a layer of amorphous flocculent material could be seen adjacent to the endothelium in some capillaries. Ruthenium red is an organic dye that binds to and precipitates several acid mucopolysaccharides including epithelial mucus and heparin.

Until the advent of the electron microscope, the presence of a basement membrane had not been certainly established. On electron-micrographs a definite membrane is seen, with a characteristic fibrillar structure and a uniform thickness in the order of 500 Å.[23-30] It was reported that the bulk of the membrane consisted of collagen rather than, as often supposed, mucopolysaccharides.[30] This might explain the lack of sucess when attempts have been made to increase capillary permeability with hyaluronidase. It seemed that the basement membrane acted as a microskeleton, often persisting after the loss of endothelium due to pathologic processes. It also acted as a filter, this being seen where the main filtration barrier, the endothelium, was absent. It would retain particles larger than about 70 Å in diameter.

Completely enclosed in the vascular basement membrane were the pericytes.[30] They were spidery cells hugging the smallest vessels and usually represented in electron micrographs

by one or more fragments of cytoplasm applied against the endothelium, a nucleus being only rarely seen. They had some of the characteristics of smooth muscle and were phagocytic.[31] According to Movat and Fernando,[28] the pericytes could become detatched and perhaps develop into macrophages.

In common usage, venules up to 50 micra in diameter are often classed as capillaries, but since there was good evidence that the finest roots of the venous system (8 - 100 micra in diameter) behave differently from capillaries, Majno[30] considered that they should be regarded as separate entities.

The fine structure of the pulpal vessels

Mathews et al[32] studied the fine structure of the dental pulp with particular emphasis on the blood vessels, the nerves and their relationship. The arterioles and venules were thin walled and did not exceed 100 micra in diameter. The description of capillary fine structure was similar to that given above.

In 1963, Han and Avery[33] reported on the ultrastructure of capillaries and arterioles in the hamster dental pulp. Again the capillary structure appeared similar to that described before. The authors noted many fine intracellular fibrils in the endothelial cells with a diameter of 60 - 70 Å. These were taken as evidence for the existence of independent capillary contractility. The endothelial junction was similar to that seen in previous studies. Special attention was drawn to the luminal tips of the adjacent cells which seemed to bend over and protect

the junction, perhaps preventing the exit of red blood cells. It was suggested that the pericytes might produce fibrils of collagen.

Haim[34] showed similar pictures but in addition his method of fixation left the luminal membranes of the endothelial cells appearing rather fuzzy, somewhat similar to the micrographs shown by Luft[29] as evidence for an endocapillary layer.

Vascular patterns and function

The skin

The dermis is extremly poor in capillaries which are present only in the dermal papillae. The rest of the dermis contains a complicated network of venules.[8] The capillary loops rise in the papillae of the corium and return to enter the subpapillary venous plexus. There is a rich nerwork of capillaries around the sweat glands, at the base of hair follicles and around sebaceous glands. A particularly prominent feature is the large number of arterio-venous anastomoses. The blood flow in the skin is greatly in excess of its metabolic requirements, being chiefly determined by the need to maintain thermal balance.[35, 36]

The dental pulp

The circulation of the pulp is also more than adequate for the life of the tooth, since the pulp can survive when one

apex of a multirooted tooth is amputated.[37] Three or four arterioles enter the apex of the tooth and then, towards the center of the pulp, branch and become larger.[17, 38] The larger vessels give rise to a peripheral plexus of smaller vessels[39] which then drain back into the central venules which are much larger and more numerous than the arterioles.[37, 38] A remarkable feature of these venules is the considerable reduction in size they undergo as they pass through the apical foramina.[38, 39] Arterio-venous connections have been demonstrated in the pulp by Kramer[40] and by Bennett et al.[41] The diameters of the blood vessels are consistently smaller in the pulps of deciduous teeth than in those of permanent teeth.[38]

The relation of the capillary plexus to the layer of odontoblasts has been the subject of much discussion. Boling[37] noted many fine capillary loops in the odontoblastic layer with a second plexus beneath it. Boling studied material taken from cats and dogs, and Warwick James[42] demonstrated a similar close relationship between the capillaries and the odontoblastic layer in the pulps of teeth taken from a variety of other mammals as well as reptiles, amphibia and fishes. He drew attention to the nutritional importance of this arrangement.

Adams,[43] in 1959, examined injected rodent incisors and found a very close meshwork of capillaries adjacent to the predentine and lying among the distal ends of the odontoblasts. A looser network was found in the subodontoblastic region. Adams suggested that this arrangement may be related to rapid dentine formation.

Bernick,[44] the following year, reported a study of the vascular supply to the developing teeth of rats. In the molars he found that the capillaries in the coronal area lay immediately adjacent to the developing predentine. The plexus became less and less involved with the odontoblasts toward the apical region until, at the base of the tooth, it was limited to the basal surface of the odontoblasts. In the incisors he found that, at the apex, vessels were external to the odontoblasts but towards the tip of the tooth they could be seen entering the odontoblastic zone.

Kramer[40] found that in the human dental pulp the peripheral plexus was mainly subodontoblastic. A small number of capillary loops penetrated the odontoblastic layer. In 1962, Castelli[45] found that in adult cats the main part of the peripheral plexus was subodontoblastic with a few loops going into the odontoblastic layer but not reaching the predentine.

The same year, Bernick[46] found that in rats under four months old the terminal vascular bed of the pulp was located at the surface of the predentine. As the animals became older, there was a gradual 'withdrawal' of the terminal vascular plexus from the odontoblastic zone so that, at eight months of age, all the vascular terminals were restricted in location to the pulpal-odontoblastic border.

In 1962, Adams[47] found that where there was rapid dentine formation in the teeth of young cats there was also a dense network of capillaries near the predentine. However, as the odontoblastic layer became thicker and dentine production slowed down, the main plexus came to lie on the pulpal side of

the odontoblasts. Bennett et al[41] showed similar findings in the human dental pulp. As age increased, the number of vascular structures in the pulp decreased, primarily at the expense of vessels in the subodontoblastic layer.

As long ago as 1931 Fish[48,49] stated that, under experimental cavities in the teeth of monkeys, the capillaries invaded the odontoblastic layer and came to lie between the injured dentine and the odontoblasts. Secondary dentine formation followed. Seltzer and Bender[50] stated that capillaries are always present in the odontoblastic layer but are often not visible until the pulp becomes inflamed.

Vascular permeability

Before considering the changes that take place in the vessel wall during acute inflammation, it would be best to describe the mechanisms involved in the transport of substances across the normal vessel wall. The two main possibilities are either that the endothelium is involved in active secretion of material or that it acts as a passive membrane and the transport of molecules across it obeys basic physico-chemical laws.

The second of the two mechanisms is the one commonly accepted and was first proposed by Starling[51] in 1896. He postulated that although water and salts in the blood were freely diffusible through the vessel wall, plasma proteins were not, the concentration of these being about eight percent in the blood but only two to three percent in the lymph. Starling[52] found by experimentation that the osmotic pressure of the serum proteins was small, but of the same order as capillary blood pressure:

30 to 40mm of mercury. His hypothesis was that capillary pressure determined transudation while the osmotic pressure of the serum proteins determined absorption. The endothelium need have no active role, simply acting as a passive membrane permeable to small molecules but not large ones.

Starling's hypothesis recieved experimental support when Landis[53] perfected his method of measuring the blood pressure in small blood vessels. He used minute microneedles to enter and measure the blood pressures in the smallest vessels of many different tissues in a variety of animals. He summarised his comprehensive studies, in 1934.[54] They confirmed, in general, the correctness of Starling's hypothesis and showed that the three main factors affecting the movement of fluid through the capillary wall were physical. The three factors were: 1. Capillary blood pressure favouring fluid loss from the vessel. Owing to the pressure gradient, filtration was favoured on the arterial side while absorption was favoured on the venous side. 2. The colloid osmotic pressure of the bloodfavouredfluid retention due to the relative impermeability of the capillary wall to plasma proteins. 3. Tissue pressure would tend to encourage retention of fluid in the vessel.

Pappenheimer[55] and his colleagues performed many experiments on capillary permeability and he summarised their results in 1953. He concluded that their findings and those of others could best be explained on the assumption that water and lipid-insoluble molecules including plasma proteins left the vessels by diffusion through water filled channels or pores of fixed dimensions located in the vessel wall. The pores

need not be uniform in size but his calculations showed that the passage of material through the walls could be accounted for by the presence of uniform cylindrical pores of radius 30 to 45 Å and occupying less than 0.2 percent of the surface of the endothelium.

The pore size calculated would be sufficently great to allow even large plasma protein molecules to penetrate the capillary walls. To explain the molecular sieving which occured (the passage of smaller proteins such as albumin more rapidly than the larger globulins), he introduced the concept of restricted diffusion. This meant that two forces hampered the flow through the pores in the capillary wall: viscous drag between the molecule and the walls of the pore and steric hindrance (obstruction to the passage of the molecule if it impinges on the margins of the pore before entering).

For small molecules, the restriction to diffusion was small and no appreciable gradients were maintained across capillary membranes. For large molecules, the restriction to diffusion becomes so great that the degree of molecular sieving is determined largely by the rate of filtration.

Grotte[56] came to similar conclusions but, in addition, suggested that as well as the pores there is a smaller number of larger 'leaks' affording no selective restriction to molecules with molecular weights up to at least 300,000. Mayerson et al[57] agreed with this, but thought that these larger pores allowed the passage of molecules with molecular weights up to at least 412,000.

Landis and Pappenheimer[58] have recently reviewed the subject of capillary permeability and gave a composite formula for the filtration of fluid through the capillary wall:

F.M. = k(capillary blood pressure - plasma osmotic pressure - interstitial pressure + interstitial osmotic pressure)

where + = filtration and - = absorption,
F.M. = fluid movement and k = filtration coefficient.

The figure calculated for these pressures supported the Starling filtration-absorption principle and the view that the endothelium acted as a semipermeable membrane with pores in it with a radius of 41 to 44 Å. These pores were, of course, merely hypothetical but the calculated dimension coincided very well with the width of the endothelial junction as seen on electron micrographs.

Luft[29] has proposed that the permeable channels are found at the endothelial junctions. He suggested that fluid movement takes place at the intercellular junctions through the middle lamella of the tight junction itself. This central layer is composed of the external leaflets or layers of the two adjoining cell membranes. The tight junction is the morphologic equivalent of the 'slit' in Pappenheimer's model, the walls of the slit being defined by the lipid layers of the two unit membranes. The dimensions of the tight junction as seen on electron micrographs were compatible with the values calculated by Landis and Pappenheimer.[58]

This narrow slit would account for the passage of small molecules across the vessel wall but the passage of larger molecules was thought by Landis and Pappenheimer to be accounted for by larger leaks as proposed by Grotte.[56] Landis[59] has compared the passage of a rapidly diffusible dye (patent blue V) with that of a slowly diffusible one (Evans blue) through the walls of blood vessels in the mesentery of the frog. He found that there was a generalized and uniform passage of patent blue V, revealing the general and uniform distribution of a set of openings corresponding to the pores and slits postulated by Pappenheimer.[55] In contrast to this there was a spotty passage of Evans blue occuring mainly on the venular side of the microcirculation. Landis maintained that this indicated a sparse population of larger openings with a diameter of 250 to 500 Å, increasing in number toward the venular side. Luft[29] suggested that these larger leaks probably occured at the points where three or more endothelial cells intersected.

Permeability of the vessels of the dental pulp

In a series of reports Haldi et al[60, 61, 62] analysed the constituents of the dental pulp fluid in relation to the blood plasma. They drilled holes in the buccal enamel and dentine through to the pulp chambers of the canine, premolar and molar teeth of dogs. Glass capillary tubes were inserted into each hole and sealed into place. After several hours the tubes were filled with a clear colourless fluid which was designated as dental pulp fluid. They claimed that the procedures performed

on the teeth caused no damage to the pulp. Plasma was obtained from venous blood drawn towards the end of each experiment.

It was found[60] that proteins were present in the same relative concentrations in the dental pulp fluid as in the plasma, except that albumin was present in a slightly higher relative concentration in the dental pulp fluid. The absolute amounts of the protein fractions was much higher in the plasma than in the dental pulp fluid. Analysis[62] of chlorides, magnesium, urea, and alkaline phosphatase in the blood plasma and dental pulp fluid revealed no significant differences in the concentrations in the two fluids. Calcium and inorganic phosphorous were at a significantly higher concentration in the plasma than in the pulp fluid, indicating that a portion of the inorganic calcium and phosphorous in the blood plasma is non-diffusible. The glucose concentration[61] in the dental pulp fluid approximated the concentration in the blood plasma and rose and fell with changes in the level of glucose in the plasma. These results led Haldi et al[62] to the conclusion that the dental pulp fluid was a capillary filtrate.

Vascular permeability in acute inflammation

As noted at the beginning of this review, one of the remarkable early changes in the process of inflammation is the alteration in the permeability of the vessels. This was one of the main observations made by Cohnheim[12] who referred to what he supposed was a 'molecular change in the vessel walls'.

In 1927, Lewis,[63] having made extensive investigations into the reactions of the peripheral vessels to injury, first described what he called the triple response: local vasodilatation, the flare and local edema. He showed that the edema was mainly due to an increase in the permeability of the vessels. He provided good evidence that injured tissues released a substance similar to histamine, which acted upon the surrounding vessels and caused them to become abnormally permeable.

Landis[54] found that the increase in capillary permeability to colloids was the fundamental reason for all forms of local and general edema produced by injury. The edema was also enhanced by increased capillary blood pressure due to vasodilatation and temporary blockage of capillary flow due to stasis. The effect of tissue injury was to increase the filtration coefficient up to seven times.[58] Some investigators have found that the concentration of protein in edema fluids during inflammation is essentially that of the plasma.[12, 54, 64] The normal concentration of protein in the lymph is less than a quarter of that in the plasma.

During the last few years it has been noticed that injury can cause three types of leakage. Cotran and Majno[65] described and compared them at a recent symposium. Histamine, serotonin, bradykinin and the Miles factor induce an early transient response, vascular leakage beginning immediately and lasting less than 30 minutes. The leaking vessels are predominantly venules which partially dissociate along the borders of the endothelial cells. The plasma escapes through these channels and filters across the basement membrane.[66]

The mechanism causing this increased permeability is unknown. Spaces appear between the venular endothelial cells to permit the loss of excess fluid. Haddy[67] and Rowley[68] produced evidence that histamine produces increased permeability by causing constriction of the small veins and so increasing blood pressure in the venules. Two objections have been raised to this view of events. Majno[30] has applied a pheumatic cuff to the hind limb of a rat to produce an increase in venous pressure. He could not find evidence that this produced vascular leakage similar to that caused by histamine. Landis and Pappenheimer[58] pointed out that histamine produces edema fluid containing four to five percent protein whereas, if venous pressure is raised by other means, the filtrate contains at the most 0.7 percent protein.

Spector and Willoughby[69] have suggested a number of possible mechanisms by which histamine could increase capillary permeability: contraction of the endothelial cells causing them to draw apart and form gaps; an alteration in the permeability of the cells causing them to round up and separate; or an alteration in the active transport system of the cells.

The second type of vascular leakage considered by Cotran and Majno[65] was that caused by direct vascular injury. All vessels are affected, the endothelial cells being swollen and disrupted and, in places, even absent. The basement membrane reveals a high degree of resistance and persists even if the endothelial lining is largely destroyed.

Thirdly, if certain injurious agents such as heat, ultraviolet rays, X-rays, bacteria or bacterial toxins are applied in

suitable doses, it is possible to induce a vascular leakage which sets in with a delay and lasts for several hours. A suitable agent is mild heat (54° C for 20 seconds), and it is apparent that the vessels which leak the most are the capillaries of the superficial network. In some lesions they are the only vessels to leak, while in others there are some leaking venules. The localization of the leakage at the level of the capillaries is remarkable when contrasted with the venular leakage pattern obtained with histamine. The leakage is due to the formation of gaps between endothelial cells, similar to those induced in venules by histamine.

Vascular labelling

Recent studies on vascular permeability have used a method now known as 'vascular labelling'. This is not a new phenomenon but its significance was only clarified following electron microscopic studies by Majno and Palade[70] in 1961.

Hertzog,[71] in 1925, injected Chicago blue dye and India ink intravenously and found that in injured areas, the walls of the capillary vessels allowed the dye to escape, while the ink accumulated in the vessels, eventually obstructing the egress of the dye.

Krogh[72] formulated the theory that the increase in the permeability of vessels was caused by the formation of fissures between endothelial cells, especially where the borders of three or more cells would meet. To test this hypothesis, he used filtered and dialysed India ink added to the blood so as to

make the plasma a distinct grey colour. He assumed that if he were correct and microscopic openings in the wall were formed in leaking vessels, this grey plasma would escape into the surrounding tissue. However, the result was that the India ink particles were held back while only clear plasma escaped. As a result of this finding he was forced to reject his hypothesis.

Majno and Palade[70] performed similar experiments, causing localized leakage by the application of histamine and serotonin to the cremaster muscle of the rat. With the electron microscope they showed that Krogh's[72] theory was correct. The pictures suggested that the endothelial cells became partially disconnected along the intercellular junctions. These openings were seen to be filled with plasma and with ink particles which had not escaped into the surrounding tissue since the basement membrane was usually intact. The accumulation of tracer particles against it indicated that it acted as a filter, allowing fluid to escape but holding back suspended particulate matter. Krogh was unaware of the presence of the basement membrane and it was responsible for his failure to prove an hypothesis which later was shown to be correct.

The results of Majno and Palade[70] have been confirmed by Marchesi[73] and by Cotran.[74] Since 1961 many studies have been made using the method of 'vascular labelling', which is used to maximal advantage when the tissues are cleared and examined with the light microscope. If an hour or two is allowed to pass following the intravenous injection of the India ink it is found that all the carbon particles are removed from the blood

by the reticulo-endothelial system. Leaking vessels can then be accurately identified in relation to the whole vascular tree since they are blackened due to the carbon particles lodged in the vessel wall.

The method was used by Majno et al[66] to show that the leakage due to histamine occured mainly from the venules. Cotran and Majno[75] used it to show that after moderate thermal injury, most of the leakage was from the capillaries. Rowley[68] used 'vascular labelling' to demonstrate the increase in permeability due to histamine and other mediators. Hurley[76] showed that when leukocytes were emigrating from small vessels, no carbon escaped with them because the endothelial cells formed a tight seal around the emigrating cell. If an increase in permeability was induced at the same time, however, the carbon particles passed down the endothelial junctions and then out of the vessels,since the emigrating leukocytes seemed to have altered the ability of the basement membrane to arrest their passage.

In 1965, Hurley and Spector[77] used colloidal carbon to study the pattern of increased vascular permeability in the diaphragm of rats with turpentine-induced pleurisy. They found that, initially, only venules seven to eighty micra in diameter leaked and that this leakage was inhibited by small doses of antihistamines, pointing to the role of histamine as a mediator in this early response. After 45 minutes some capillaries four to six micra in diameter were leaking as well and at two hours they were the only vessels leaking. At five hours however, the venules were once again labelled, this being suppressed by systemic

doses of salicylate. Salicylate is known to inhibit the major part of exudate formation. At no time was the labelling of capillaries suppressed by antihistamines or salicylate. The results supported the view that with the injury mentioned above, increased vascular permeability is due to two mediator systems operating in sequence: the first histamine and the second uncertain but inhibited by salicylate. Leakage from capillaries could be due to direct damage or to an indirect mechanism as yet obscure.

The previous study was followed up by Ham and Hurley[78] who made an electron-microscopic study of terpentine-induced pleurisy in the rat. They found that in this experimental system carbon deposition was a true indicator of increased venular permeability, but that in capillaries most of the carbon lay in the lumen of the vessels and indicated thrombus formation following damage to the endothelial cells. In capillaries carbon deposition did not indicate an increase in permeability.

Histamine and vascular permeability

The literature regarding histamine is voluminous and the reader is referred to the reviews by Spector and Willoughby[7, 69, 79] for a full account of the substance and its relation to the events occuring in inflammation.

Histamine was first demonstrated and its pharmacologic properties described by Dale and his co-workers,[80, 81] in 1910. It is formed by decarboxylation of the amino-acid histidine in the presence of the enzyme histidine decarboxylase and is found in most tissues and physiologic fluids. Under normal circumstances

it is found in the tissues closely associated with mast cells.[69]

Briefly, its properties are that it: causes marked dilatation of capillaries and increases permeability to plasma protein in the venules in preference to the capillaries while on the arterioles it has a selective action according to the species. Thus in rodents there is a marked constriction of the arterioles under the influence of histamine, in cats only a slight constriction and in the dog, monkey and man a dilatation of arterioles.[69] The net result in all cases is a fall in the systemic blood pressure. Histamine stimulates smooth muscle, but with a marked species difference.

Histamine induces an immediate but short lived increase in venular permeability, usually lasting no longer than 30 minutes.[65, 66, 68, 77] The mechanisms by which it brings this about have been discussed above.[69] Histamine also seems to be related to the immediate, short lived increase in permeability caused by many non-specific irritants. It is not related to any later or longer lasting increase.[77]

Histamine is loosely bound to the granular matter of the mast cell, presumably by weak bonds to anionic groups such as carboxylates.[82] Non-specific irritants as well as antigen-antibody reactions and histamine liberators such as 48/80 appear to act on the mast cell to cause the release of histamine.[69, 79, 83, 84] Thus histamine is thought to be the mediator which causes the early, brief increase in permeability seen in turpentine-induced pleurisy,[77] in antigen-antibody reactions[85] and when histamine liberators are administered.[68]

Vascular changes in the dental pulp

Pohto and Scheinin[1] developed a method for observing the living pulp of rat incisors. The alveolar bone over the tooth was removed and the tooth ground down on both sides so that the pulp could be observed through a thin layer of dentine. They could watch the changes that the vascular system of the pulp underwent following various stimulants such as heat and the application of eugenol. Hyperemia occured, often causing an increase in the rate of flow in the vessels but only a slight increase in size. Trypan blue was injected intraperitoneally into the rat on the night before the experiment. It was found that in areas of the pulp which had been damaged, blue staining occured indicating a leakage of plasma from the vessels.

Other investigators have observed material thought to be exudate in histologic sections. Seelig and Lefkowitz[86] considered that the eosinophilic material in the pulp tissue underlying the cavities cut in the teeth of monkeys was blood protein which had diffused through the capillary walls as a result of inflammation. Kramer and McLean[87] observed the same phenomenon but did not use it as a criterion for assessing pulp damage. Mohammed and Schour,[88] Marsland and Shovelton,[89] Zander[90] and Swerdlow et al[91] all agreed that the eosinophilic material under experimental cavities was an exudate of plasma. Ingle and Ogilvie[92] attributed the staining to a chemical alteration of the ground substance. However both they and Seltzer and Bender[50] stated that there is an increase in the permeability of vessels in the pulp nearest the site of injury allowing fluid to escape into the connective tissues.

Beveridge and Brown[2] have shown that the intrapulpal pressure is influenced by temperature changes and cavity preparation. Heat increased the pulpal pressure whilst cold reduced it. There was no well defined pattern of intrapulpal pressure response during either drilling or grinding on superficial layers of the tooth. Changes were more pronounced as tooth removal progressed nearer the pulp. When a carbide bur was used in an airotor without a coolant to cut the cavities, the intrapulpal pressure dropped at first and then after a few seconds returned to the starting level and rose above it. This may be related to the findings of Zach and Cohen[93] and of Bhasker and Lilly[94] that when cavity preparation was carried out with an airotor without coolant, the temperature in the pulp chamber decreases for the first ten seconds followed by an increase in temperature. It was considered significant[2] that changes in pressure were not found upon pulp exposure when the site of exposure was more than a few millimeters from the site of the pulpal tap. This finding implied that pressure is not easily transmitted from one part of the pulp to another.

Dachi[4] studied a hundred teeth which had had experimental cavities cut in them and amalgam and silicate restorations placed. Sensitivity to heat and cold was tested seven days after cavity preparation by holding ice or hot gutta percha against the cervical region of the tooth for five seconds. The teeth were then removed and decalcified sections made. From these the degree of inflammation and the degree of hyperemia was evaluated subjectively. It was found that the number of teeth sensitive to both cold and heat increased significantly in the presence of

hyperemia. Sensitivity to heat was also related to the degree of inflammation but sensitivity to cold was not. There was some relationship between the presence of hyperemia and the presence of inflammation but since there were very few teeth free of inflammation it was not possible to ascertain if pulpal hyperemia could occur without inflammation.

Scheinin[95] stated that his observations on the incisor of the rat showed that in hyperemia of the pulp there is a marked increase in the rate of flow but little increase in the size of the vessels, while dilated vessels were often seen when there was a retardation of flow due to local analgesics. He therefore concluded that the seemingly widened vessels filled with erythrocytes, seen in histologic sections, should not be considered to indicate hyperemia.

STATEMENT OF PROBLEM

The purpose of this study was to compare the permeability of the vessels in the dental pulp and in the skin during acute inflammation. It was considered that it would be useful to find out which vessels leaked plasma during acute inflammation and also the quantity of plasma leaked.

The problem was to adapt two methods, previously used in the study of vascular permeability in the skin[74, 75] and in the liver,[96] for use in studying vascular permeability in the dental pulp.

EXPERIMENTAL PROCEDURE

Preliminary trials

Preliminary trials were conducted using rats as the experimental animals, in an attempt to find the best method for causing moderate injury to the skin and dental pulp and to discover if the experimental methods gave results in the author's hands.

To cause moderate heat injury to the skin a heat pulp tester, designed and used by Jennings,[97] was adapted for use. it consisted of a small soldering iron with a flat circular tip 2mm in diameter. Power for the iron was supplied at a constant voltage from a black box containing a voltage regulator and a variable resistor. This variable resistor was connected in series with the iron and served to control the voltage across the iron. Thus the temperature of the tip of the iron could be regulated by this device.

Calibration of the instrument was undertaken using an iron-constantan thermocouple with a cold junction in a vacuum flask filled with ice. The voltage across the thermocouple could be read directly using a Sandborn Carrier preamplifier and recorder. The temperatures of the room and of the cold junction were measured with mercury thermometers. Taking these temperatures into account the temperature of the hot junction, and thus of the tip of the iron,could be calculated from the voltage across the thermocouple.

The preliminary trials with carbon labelling will not be described as they were successful and the method will be described in full with the principle experiment. The aim of the method was to indicate the location of leaking vessels in inflamed areas.

An attempt was made to estimate the quantity of leaking plasma by using bovine globulin conjugated to a fluorescent dye. This method has previously been used in the study of experimental liver damage.[96] A conjugate of bovine globulin and the fluorescent dye lissamine rhodamine B. 200 (R. B. 200 or sulphorhodamine) was prepared according to the method described by Nairn.[98]

A gallon of bovine blood was obtained from the Hygrade Meat Company, Indianapolis and allowed to stand for 48 hours in a cold room (less than 4^{o} C). It was then possible to decant 500ml of serum from the contracted clot. The following procedures were all carried out a temperature below 4^{o} C. The globulin fraction of the plasma proteins was obtained by precipitating with ammonium sulphate; 500ml of a 50 percent saturated solution. This crude globulin fraction was removed by filtration and dissolved in 300ml of phosphate buffered saline. This was physiologic saline buffered at pH 7.1 with 0.01 molar phosphate and was prepared by dissolving 8.5 grams of sodium chloride, 1.07 grams of anhydrous sodium phosphate and 0.39 grams of sodium biphosphate in one litre of distilled water. The solution of globulin was dialysed for four days against distilled water, in order to remove the ammonium salt, and the globulin obtained as a fluffy powder by freeze-drying.

A preparation of the sulphonyl chloride of R. B. 200 was made by grinding 3.5 grams of lissamine rhodamine B. 200 together with 7.0 grams of phosphorous pentachloride in a mortar until thoroughly mixed and then dissolving the mixture in 35ml of acetone. This solution was added slowly over a period of 30 minutes to a solution of globulin prepared by dissolving 18 grams of globulin obtained from the previous procedure to 100ml of carbonate-bicarbonate buffer. The temperature of the reactants was at all times kept below 4^{o} C. The carbonate-bicarbonate buffer was prepared by dissolving 3.7 grams of sodium bicarbonate and 0.6 grams of anhydrous sodium carbonate in sufficient distilled water to make 100ml of solution and had a pH of 9.0. Following the addition of the fluorochrome the mixture was stirred for another 30 minutes and then dialysed for two days against distilled water in the cold room.

A column was prepared to remove the unreacted fluorochrome from the conjugate of protein and dye. A cross-linked dextran, Sephadex G-25, was allowed to swell in phosphate buffered saline for six hours and then placed in a column 25cm tall with a diameter of 3cm. A small quantity of the dialysed mixture was placed at the top of the column and washed through with phosphate buffered saline. The conjugated globulin passed through the column rapidly while the unreacted fluorochrome remained at the head of the column. The conjugated globulin was collected and freeze-dried.

Two male white rats, weight 300 grams each, were anaesthetised with intraperitoneal injections of 2ml of a ten percent solution of sodium thiopental. The temperature of the

room was 25° C and the electric pulp tester had been left on over night at a setting of 18.5 to give a temperature at the tip of the iron estimated at between 54° and 58° C. The tip was held firmly against the shaven skin of the abdomen for 20 seconds and caused a wheal to form at the site of injury during the next three to four hours. Four injuries were caused on the abdomen of each animal and two areas were left to use as comparison. A solution was prepared by mixing the globulin - R.B.200 conjugate with 3.0ml of normal saline. This solution was divided into two equal portions and injected into the caudal vein of each animal four hours after the injury was caused. Thirty minutes later the rats were sacrificed with an overdose of chloroform. The abdominal skin was dissected away from the underlying muscle, spread out with pins on a sheet of dental wax and placed in a bottle of ten percent formalin.

Half of the material from each animal was processed normally as for paraffin sections while the other half was passed through a decalcification procedure in five percent formic acid and then processed for paraffin sections. This was to determine if the fluorescence of the conjugate was lost during decalcification. Some sections from each group were stained with hematoxylin and eosin while others were left unstained and examined under the fluorescent microscope.

Principal study

Three mongrel dogs obtained from the Wilson Animal Farm, Vincennes, Indiana were used for this portion of the study. The principles of laboratory care of the National Society for Medical Research were observed at all times. The procedures performed on each animal are summarized in Table I.

Preparation of the animal for treatment: The dog was anaesthetized with sodium pentobarbital given intravenously in the foreleg. A 50 milligram per milliliter solution was used and the doseage was one milliliter per five pounds body weight. As the procedures on each animal took up to six hours to perform, it became necessary to give one to two milliliters of the narcotic later to maintain an adequate depth of anaesthesia.

The animal was placed on the operating table in a supine position, the head resting on a bag of corn to stabilize it. The hair was shaven from the abdomen and chest and a marking pencil used to divide the area into sections. The body of the animal was draped with a sheet in order to conserve heat. Room temperature was maintained at 25^{o} C.

Experimental procedures: The buccal enamel of the teeth to be studied was removed using a number two round bur in an airdriven handpiece. The rest of the cavity was prepared using a number six round bur in a belt driven handpiece. The microscope had a 0.5X objective and a 10X eyepiece; a

rotary "magnichanger" in the barrel of the microscope allowed the power of the instrument to be changed easily. The arrangement used gave a magnification of 15X and a working distance of eight inches. The slow running handpiece was used with intermittent light pressure and the assistant used an air syringe only occasionally to blow away debris. The preparation was carried out quickly until the pink colour of the pulp began to show through. Then the dentine overlying the pulp was gently cut away with the slowly revolving bur in an attempt to leave as thin a layer of dentine as possible. The procedure was similar to that employed by Hassan[3] and the time taken to prepare each tooth was five to twenty minutes. A considerable amount of fluid accumulated on the cavity floor in the deep cavities, about 15 minutes after cavity preparation was completed. This finding supported the findings of Hassan and the fluid was identified as the "dental-pulp fluid".

The pulps of some of the teeth were accidentally exposed, a few teeth had only shallow cavities prepared and some had none. The details are given in Tables II - IV. When cavity preparation was complete, certain teeth were selected and 0.05ml of histmine phosphate solution dropped into each cavity. A tuberculin syringe graduated in increments of 0.01ml was used to dispense the solution which contained 2.75mg of histamine phosphate per 5ml, with 16mg of glycerin and 2mg of phenol. A pellet of cotton moistened with the solution was inserted into the cavity to keep it dampened. Details of the teeth treated in this manner are given in Table IV.

Certain areas of the abdomen and thorax received intradermal injections of 0.01 or 0.05ml histamine phosphate solution using a tuberculin syringe with a 27 gauge needle. Other areas were left for comparison by just inserting the needle for a short time.

An intravenous injection of carbon particles was made. The suspension was prepared from carbon ink* which contained about 100 milligrams of carbon per milliliter with an indicated particle size of 200 Å.[66] The ink was stabilized with 4.5 percent fish glue and contained 1.3 percent phenol as a preservative. Unlike India ink, this preparation contained no shellac, improving it for biologic use. The phenol was removed by dialysis the night before the experiments and the ink diluted by adding an equal volume of normal saline.

Unfortunately, at a critical stage of the procedure on the third dog the dialysed ink was lost and it was necessary to use the undialysed ink. This dog went into mild spasms following the injection of this phenol-containing ink and it took longer than normal for the ink to be removed from the blood stream.

In all three dogs the ink was injected into a vein in the foreleg , the amount given varying according to the weight of the animal. The dosage given was one milliliter(100 milligrams of carbon) per kilogram weight of the dog. Thus for a 25kg animal 50ml of the carbon-saline mixture was given. Upon

* Pelikan ink(C11/1431a), Gunther Wagner, Pelikan Werke, Hanover, Germany. Agents: John Henschel and Co. Inc., 195 Marine Street, Farmingdale, Long Island, New York.

injection of the ink the skin of the animal turned black but after an hour or more, as the carbon was removed from the circulation by the animal's reticulo-endothelial system, it regained its normal colour. The only areas which remained dark to the naked eye were those which had received an injection of histamine, and inflamed areas such as the gingival attachment (Figures 3-5)

When the colour of the animal had returned to normal, the dog was sacrificed with an overdose of sodium pentobarbital and the teeth were removed surgically. Immediately upon removal of the tooth it was placed in a ten percent solution of formalin and the apex was removed with the airotor handpiece. One side of the tooth was ground down with a rotating stone wheel under a water spray until the pulpal outline could be seen.

While the teeth were being removed, an assistant dissected the skin away from the thorax and abdomen and placed the different sections in ten percent formalin. Portions of the liver, spleen, lung and a lymph node were removed from the first animal and fixed in ten percent formalin.

Treatment of material: The teeth were decalcified in five percent formic acid following which both they and the other specimens were processed in one of two ways.

One method of processing was to prepare normal histologic sections. The tissue was dehydrated and embedded in paraffin and seven micron thick sections cut. Alternate slides were stained with hematoxylin and eosin or just with eosin. It was found that it was easier to locate the carbon in the sections when no hematoxylin was used.

The second method of treating the material was to prepare cleared specimens. The tissue was first dehydrated by placing in three changes of ethylene glycol monoethyl ether. At this time the teeth were trimmed with a sharp scalpel so as to remove most of the dentine overlying the pulp. Following dehydration the tissue was infiltrated with the clearing solution, methyl salicylate. This was done by placing the specimens first in a mixture of 25 percent methyl salicylate and 75 percent ethylene glycol monoethyl ether; then in 50 percent, 75 percent, and finally pure methyl salicylate.

The skin specimens were prepared for observation by making oblique sections about 100 to 150 micra thick with a sharp scalpel. The teeth were trimmed so that they would lie flat in the clearing solution and the pulp could be observed through the pulpal floor of the cavity.

Both the histologic sections and the cleared tissues were observed by transmitted light under the microscope.

DATA

Preliminary trials

The calibration of the heat pulp tester showed that at a room temperature of 25° C the temperature at the tip of the iron reached between 54° and 58° C with the variable resistor at a setting of 18.5. However the temperature fluctuated considerably within these limits and it took about two hours for the iron to reach this temperature. As a result the instrument was only used in the preliminary trials.

The trials involving the use of the fluorescent dye conjugated to bovine globulin did not appear to be successful. In none of the sections was it possible to see fluorescence due to lissamine rhodamine B. The trials with this material were not continued since the method of vascular labelling with carbon was working satisfactorily and time was not available to continue with both methods.

Principal study

The results from all three animals were similar. The third dog received an injection of ink which had not been dialysed to remove the phenol. As a result it went into spasms and the ink was not removed from the blood as fast as in the other dogs. The specimens taken from this animal showed some carbon in all the vessels but heavier deposits were seen in injured areas, similar to those seen in the other two animals. As a result the findings from the third animal were used to support those from the first two.

No histamine applied

No cavity -- histologic sections. Histologic sections of these teeth revealed normal pulpal structure although some of the specimens showed poor fixation in the coronal portion. The odontoblastic layer was always regular except that in two teeth (Dog III -1, -3) a considerable number of cells were aspirated into the dentinal tubules. Carbon was seen intravascularly in some of the sections especially those taken from Dog III. Polymorphonuclear leukocytes were seen in many vessels sometimes in considerable numbers.

Cleared tissue. The pulpal vessels were visible in these teeth although they were often not as prominent as those in the treated teeth. There was no specific labelling of vessels observed but there was intravascular carbon seen in teeth taken from Dog III.

Shallow cavity -- histologic sections and cleared tissue. There were only three teeth in this group. The histologic sections showed much the same picture as the teeth with no cavities. The odontoblastic layer appeared regular except in one tooth where it was distorted and cells displaced into the dentine underneath an area where the tooth had been traumatized on removal. The cleared specimen showed vessels particularly prominent in the apical region and below the cavity. There were a few labelled vessels, five to nine micra in diameter, just below the cavity.

Deep cavity -- histologic sections. In these teeth the odontoblast layer was disrupted and displaced slightly under the cavities, the floors of which were 25 to 30 micra thick. There was evidence of an eosinophilic substance in the cavities. This was taken to be the dental pulp-fluid which had been seen to well up in the cavity 15 or more minutes after preparation. Small vessels were seen in the odontoblastic layer under the cavities but were not visible in other regions of the layer. Marginating polymorphonuclear leukocytes were observed in the larger vessels (20 to 30 micra diameter) under the cavity. Some of the vessels under the cavity were labelled with carbon but this was difficult to see in the stained sections. A few of the small vessels under some cavities seemed to be filled with carbon particles. The specimens taken from the third dog showed some intravascular carbon in most of the vessels.

Cleared tissue. The vessels of the pulp were obvious in these teeth but labelling of vessels was confined to the region of the cavity. The labelled vessels were fairly large, being 20 to 40 micra in diameter. In three teeth there was also a fine lacework of smaller vessels, eight to ten micra in diameter, lying beneath the cavities. There was an intense deposit of carbon in these teeth.

Exposure -- cleared tissue. The extent of the carbon deposition depended on the severity of the exposure. In two teeth the whole of the coronal pulp was filled with carbon. In the other teeth there was a group of small (eight to ten micra) vessels under the cavity labelled with carbon. Beneath these there were a few larger vessels labelled to a lesser degree.

Histamine applied

Shallow cavity -- histologic sections and cleared tissue. There were only three teeth in this group. The histologic sections showed inadequate fixation and were not evaluated. The cleared tissue showed no labelling of vessels; the cavities had floor depths of 200 and 350 micra.

Deep cavity -- histologic sections. In these sections it could be seen that the odontoblastic layer under the cavities was slightly disrupted and cells displaced into the dentine. The cavity floor depth was between 35 and 50 micra. Labelled vessels were seen under the cavities in the eosin stained sections.

These were usually 20 to 30 micra in diameter and the smaller vessels nearer the cavity floor were usually not labelled. In some sections the area under the cavity contained an eosin stained region. The area under the cavity also contained many polymorphonuclear leukocytes both in the vessels and in the surrounding tissue.

Cleared tissue. The teeth in this group showed much variation. Some of them showed no labelling at all but most showed labelling of the larger (20 to 30 micra) vessels under the cavity. There was occasional labelling of the small vessels, eight to ten micra in diameter, which lay immediately below the cavities. While there was no difference between the teeth in this group and the teeth in the group which did not receive an application of histamine there was a tendency for the labelling of vessels to be seen beyond the cavity outline when histamine had been applied.

Exposure -- histologic sections. In both the teeth in this group there was only slight disruption to the pulp underneath the exposures. Carbon had escaped from ruptured vessels into the odontoblastic layer and there was a large eosinophilic area in the same region.

Cleared tissue. Many vessels were labelled in the coronal region and labelling was not confined to the cavity outline. In some teeth there appeared to be rather indistinct areas of carbon, outside the vessels, and these were taken to be regions where the vessels had ruptured allowing carbon to escape into the surrounding tissue.

Skin -- histologic sections. The untreated areas showed a normal appearance for the skin of the dog. Areas which had received an injection of histamine showed a similar picture except that the subepithelial layer showed some vacuoles and was generally swollen. Also, in the sections which had been stained with eosin, carbon could be seen in both large and small vessels.

Cleared tissue. No labelled vessels were seen in the untreated areas but there was considerable deposition of carbon in the areas where histamine had been injected. The area of labelled vessels was larger in the tissue where 0.05ml of histamine solution had been infiltrated than in the tissue where 0.01ml had been used. The number of labelled vessels was much greater, and the area covered was much larger, in the skin than in the dental pulp.

Reticulo-endothelial system. The histologic sections of tissue taken from the liver, spleen, lung, bone and lymph node showed varying amounts of carbon accumulation. It seemed that the greatest amount of carbon was located in the spleen where it was especially prominent around the area of the lymphatic white pulp and the penicilli. Under the highest power objective (X100 oil immersion) the carbon could be seen to be located inside the cytoplasm of the macrophages. The liver contained many clumps of carbon particles located in the Kupffer cells around the smaller spaces. The Kupffer cells around the larger spaces were often free of carbon. The bone marrow, lung and lymph node showed only moderate amounts of carbon taken up by the macrophages, the least amount being in the lymph node.

TABLES

TABLE I

Outline of procedures performed on the experimental animals

Dog no.	Sex	Weight	Quantity of anaesthetic* Initial	Maintenance	Time from initiation of anaesthesia: Cavity prep. started	Histamine applied	Carbon given		Sacrificed†	
I	Male	10.4kg	5ml	1ml	60min	250min	265min	46ml	360min	6ml
II	Male	11.3kg	5ml	2ml	15min	150min	160min	50ml	285min	7ml
III	Female	8.6kg	4ml	1.5ml	40min	205min	215min	38ml‡	375min	5ml

Room temperature 25° C

*Sodium pentobarbital 50mg/ml

†Sacrificed with an overdose of sodium pentobarbital, quantity shown

‡The ink used for this animal had not been dialysed (see text)

Preface to Tables II - IV

Roman numerals refer to the dog from which the teeth were taken. The numbering system for the teeth follows the Scandinavian method. The teeth in each quadrant are numbered from 1 to 10 in the upper arch and from 1 to 11 in the lower, starting at the mid-line. The quadrant is indicated by a plus(+) or minus(-) sign placed either before or after the number. The sign is imagined to be in the midline, (+) being for the upper arch and (-) for the lower. Thus the upper right fourth premolar is designated as 8+ and the lower left first molar is designated as -9.

TABLE II

Results in teeth in which no cavities were cut

Histologic sections		
I	+7	Poor fixation, some margination of polymorphs.
	-4	Normal appearance, no labelling, a few polymorphs.
III	1+	Normal appearance, some intravascular carbon.
	2+	Normal vascular pulp tissue, poor fixation coronally.
	3+	Normal pulp, with some intravascular carbon and polymorphs.
	-1	Normal pulp, with some intravascular carbon, a few displaced odontoblasts.
	-2	Poor fixation, normal pulp.
	-3	Normal pulp with some intravascular carbon, a few displaced odontoblasts.
Cleared tissue		
I	-1	No vessels visible.
	-2	A few vessels visible, no labelling.
	-6	A few vessels visible, no labelling.
III	4-	Carbon seen in vessels, no specific labelling.
	6-	Carbon in vessels, no specific labelling.
	7-	Carbon in vessels, no specific labelling.
	8-	Carbon in vessels, no specific labelling.

TABLE III

Summary of procedures and results in teeth not treated with histamine

A = the time between cavity preparation and injection of carbon particles.

B = the time between cavity preparation and sacrifice.

Shallow cavity

		A	B	
I	+1	70min	165min	Floor depth 138μ, normal pulp tissue.
	-3	80min	175min	Floor depth 200μ, normal pulp, some intravascular carbon.
	+2	90min	185min	Cleared tissue, afew small labelled vessels below cavity.

Deep cavity

Histologic sections

I	+8	60min	155min	Disrupted tissue under cavity, vessels in odontoblastic layer 25μ visible under cavity, margination of polymorphs in the larger vessels deep to the odontoblastic layer.

TABLE III (Cont.)

Deep cavity

		A	B	
Histologic sections				
III	2-	45min	205min	Floor depth 26μ, vascular odontoblastic layer below cavity, otherwise normal pulp. Some intravascular carbon.
	3-	50min	210min	Floor depth 30μ, normal pulp except for slight disruption of odontoblastic layer under cavity. Some intravascular carbon.
Cleared tissue				
I	+3	85min	180min	Pulp very vascular, labelling of a few large (20 - 40μ) vessels below cavity.
	+4	65min	160min	Pulp very vascular, a large clot fills most of the coronal pulp.
	-8	50min	145min	A few large labelled vessels (40μ) in coronal pulp, confined to cavity outline.
II	7+	40min	165min	Light lacework of small (8 - 10μ) vessels below cavity. A few larger labelled vessels deeper in the pulp.
	8+	35min	160min	Light lacework of small (8 - 10μ) vessels below cavity. A few larger (30 - 40μ) labelled vessels deeper in pulp.
	4-	-15min	110min	Many vessels visible but none labelled.
	6-	75min	200min	A fine lacework of vessels below cavity with some blotches of corbon in same area indicating ruptured vessels.

TABLE III (Cont.)

Deep cavity

		A	B	
Cleared tissue				
II	7-	65min	190min	A few larger (20 - 40μ) vessels under the cavity were labelled.
	8-	55min	180min	Vessels visible throughout pulp. A few larger vessels below the cavity were labelled.
III	4+	70min	230min	Large deposits of intravascular carbon obscured any specific labelling.
	7+	60min	220min	Labelled vessels of all dimensions obvious below cavity.
	8+	55min	215min	Specific labelling of larger vessels below cavity.

Exposure

Cleared tissue				
I	4+	190min	285min	Vessels very visible throughout pulp. A few labelled vessels below cavity (15 - 30μ).
	6+	160min	255min	Considerable labelling of vessels under the cavity.
II	4+	-25min	100min	Large mass of blood and carbon particles filled the coronal pulp.
	6+	45min	170min	Large mass of blood and carbon filled the coronal pulp.
III	6+	65min	225min	Labelling of smaller vessels below the cavity (8 - 10μ).

TABLE IV

Summary of procedures and results in teeth treated with histamine

A = the time between cavity preparation and application of histamine.

B = the time between cavity preparation and injection of carbon particles.

C = the time between cavity preparation and sacrifice.

Shallow cavity

		A	B	C	
I	1+	75min	90min	185min	Inadequate fixation.
	2+	70min	85min	165min	Cleared tissue, floor depth 350μ, many vessels in pulp but none labelled.
	2-	55min	70min	165min	Cleared tissue, floor depth 200μ, many vessels seen in pulp but none labelled.

Deep cavity

Histologic sections

I	7+	140min	155min	250min	Pulp normal except just under the cavity where there was some disruption of the odontoblastic layer.

TABLE IV (Cont.)

Deep cavity

		A	B	C	
Histologic sections					
I	4-	165min	180min	275min	Odontoblastic layer disrupted under cavity, polymorphs abundent in this area, cavity floor 50μ.
III	+2	185min	195min	355min	Cavity floor 45μ, eosinophilic substance in cavity, much intravascular carbon, otherwise normal pulp.
	+3	195min	205min	365min	Cavity floor 35μ, some intravascular carbon, eosin staining below cavity and extravasation of red cells.
Cleared tissue					
I	3+	85min	100min	195min	Many vessels visible and a few small ones just under the cavity (8 - 10μ) were labelled.
	3-	65min	80min	175min	Some labelled vessels under the cavity.
	6-	110min	125min	220min	Larger (15 - 30μ) labelled vessels deep to the cavity but not confined to the cavity outline.
II	+4	125min	135min	260min	No labelled vessels below the cavity.
	+7	90min	100min	225min	A few small (8 - 10μ) vessels labelled under the cavity, larger labelled vessels (20 - 40μ) lay below the cavity and extended apically.
	+8	85min	95min	220min	A few small labelled vessels, below them and extending away from the cavity were larger labelled vessels up to 60μ in diameter.

TABLE IV (Cont.)

Deep cavity

		A	B	C	
Cleared tissue					
II	-4	135min	145min	270min	Large vessels visible in centre of pulp, no labelling but some blotches of carbon below cavity.
	-7	105min	115min	240min	Many small labelled vessels below cavity (8 - 10μ) below these there were larger labelled vessels extending apically to the cavity.
	-8	80min	90min	215min	Small labelled vessels below cavity with larger ones extending either side of cavity. The larger vessels were labelled less intensely.
III	+4	165min	175min	335min	Labelled vessels large and small below cavity.
	+6	160min	170min	330min	Labelled vessels large and small below cavity.
	-4	105min	115min	275min	Much intravascular carbon, no labelling.
	-6	115min	125min	285min	Labelled vessels, mainly large, under cavity.
	-8	125min	135min	295min	Larger vessels only labelled, confined to cavity outline.

TABLE IV (Cont.)

		Exposure A	Exposure B	Exposure C	
Histologic sections					
I	7-	110min	125min	220min	Normal pulp except just under exposure site where there was some displacement of cells, some vessels had ruptured and carbon had escaped into the tissues.
III	+1	175min	185min	345min	Eosin staining below cavity with many polymorphs in the area. Large void just below exposure site.
Cleared tissue					
I	8-	100min	125min	220min	Numerous small labelled vessels in coronal pulp chamber, not confined to the area of the cavity.
II	+6	95min	105min	230min	Labelling of vessels under the cavity and to either side of it, also some blotching under the cavity.
	-6	115min	125min	250min	Much blotching of carbon not confined to cavity outline.
	-9	75min	85min	210min	Large labelled vessels under the cavity.
III	+7	120min	130min	290min	Labelled vessels, mainly large, under cavity.
	+8	105min	115min	275min	Labelled vessels, mainly large, under cavity.
	-7	90min	100min	260min	Many small labelled vessels under cavity, a few larger labelled vessels lying below these.

ILLUSTRATIONS

Figure 1. A diagramatic representation of a cross section of a small vessel. It shows how, when leakage is induced in the vessel, carbon (C) is swept through the intracellular junction and trapped between the basement membrane and the endothelial cell.

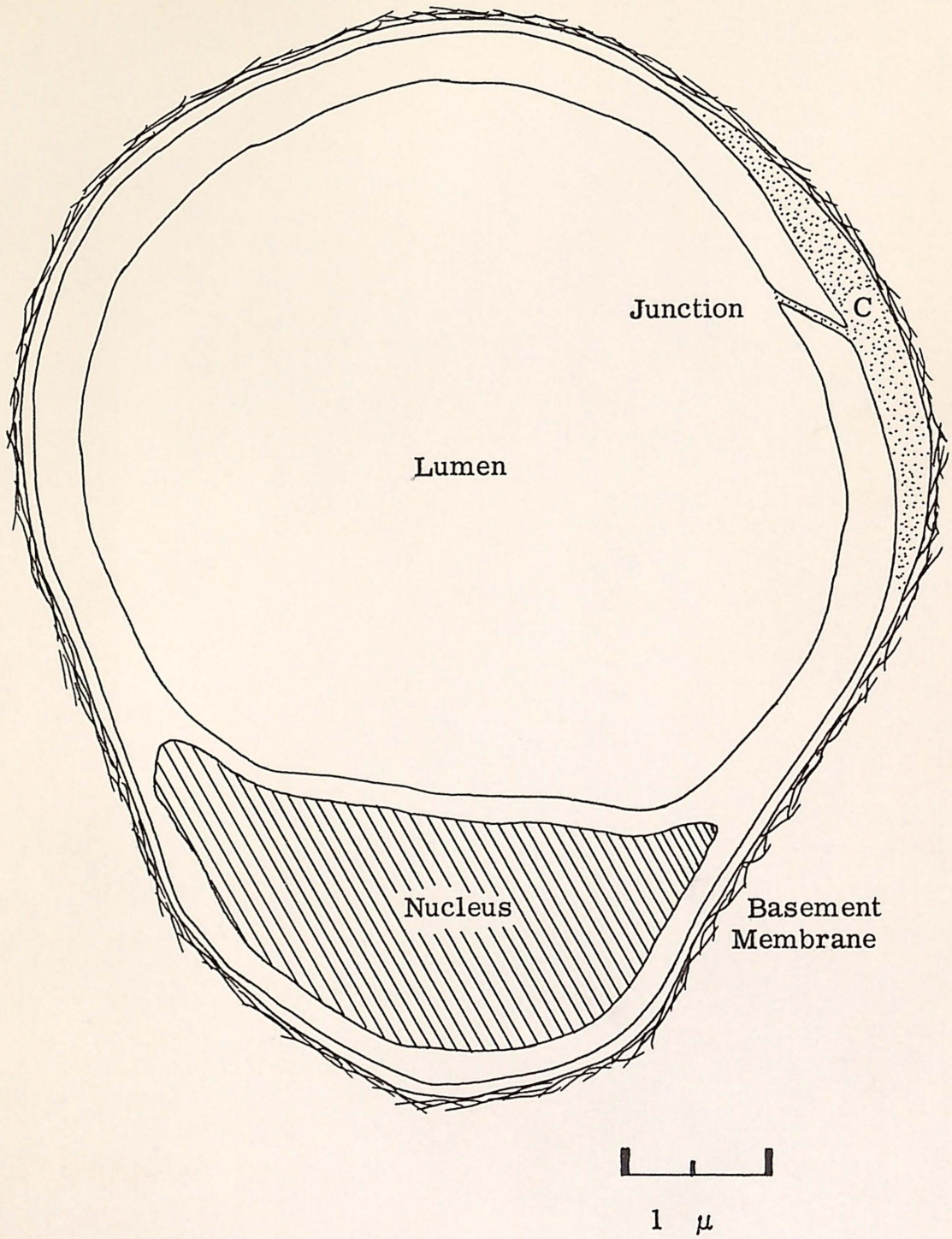

Junction
C
Lumen
Nucleus
Basement
Membrane
1 μ

Figure 2. Dog laid out in a supine position with the head tilted to one side. The animal is covered with a sheet to conserve body heat. The picture shows the "Cycloptic" microscope, the slow speed handpiece and the small lamp used for illumination.

Figure 3. Upper right canine tooth of the third dog (III 4+). A deep cavity has been cut in this tooth and the pulp can be seen through the thin cavity floor. There was no exposure either clinically or in any of the histologic sections from this tooth. Photograph taken just before the intravenous administration of carbon.

Figure 4. Dog shown in Figure 3 immediately following the administration of carbon. The carbon can be seen in the vessels of the mucosa and darkening of the pulp can be seen.

Figure 5. Dog shown in Figure 3 two hours following the administration of carbon. Note the localization of the carbon to the inflamed areas around the necks of the teeth. The rest of the mucosa is regaining its normal colour.

Figure 6. Liver, Dog number 1, Hematoxylin and Eosin. Shows the huge amount of carbon located in the vascular spaces and the Kupffer cells.

Original magnification X 215

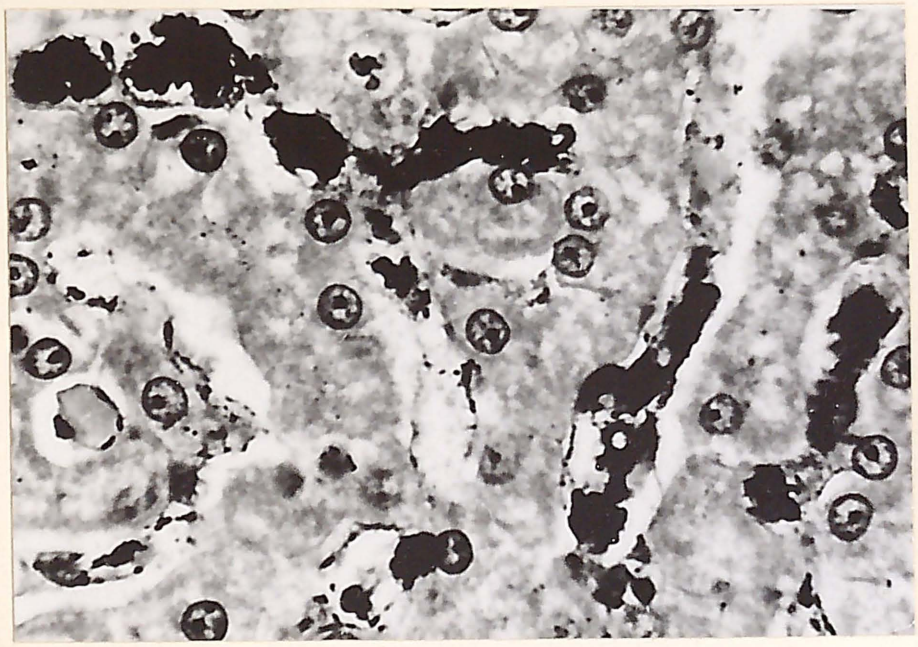


Figure 7. Cleared oblique section of skin taken from Dog number 1. This area received an injection of 0.05ml histamine phosphate solution. The labelled vessels can be seen clearly and are between 10 and 40 micra in diameter.

Original magnification X 17

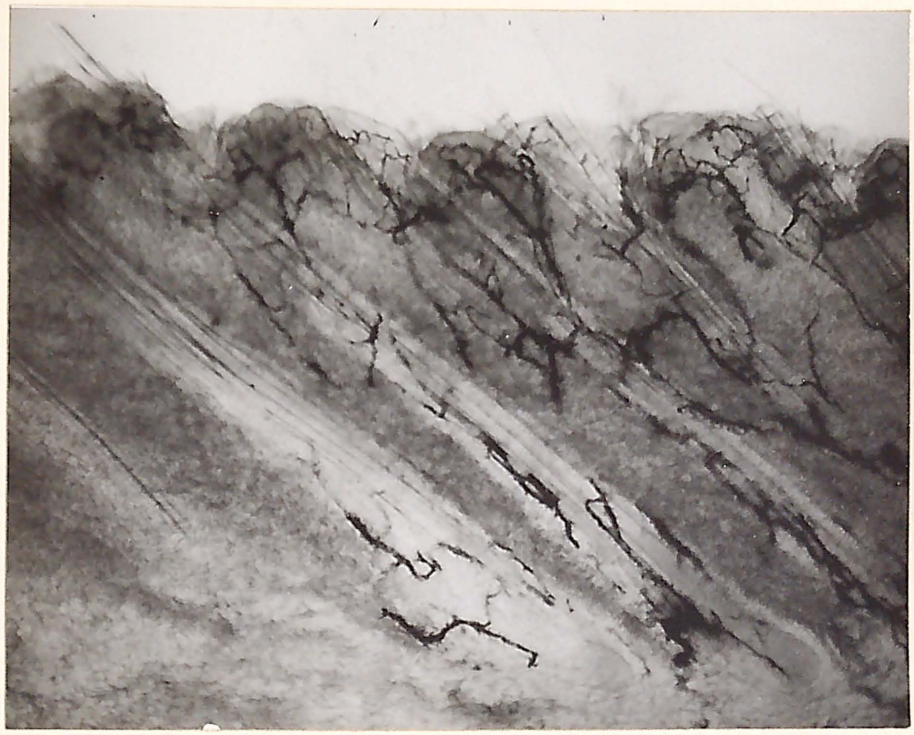


Figure 8. Dog II, tooth 6-, a deep cavity with no histamine applied. A cleared specimen viewed from the labial, through the cavity floor. The gingival floor of the cavity can be seen (arrow) as can the outline of the pulpal chamber. Many small vessels, between five and eight micra in diameter are labelled but are confined to the cavity outline. The dark blotches are areas where vessels have ruptured, allowing carbon to escape.

Original magnification X 50

Figure 9. Dog II, tooth 7+, a deep cavity with no histamine applied. A cleared specimen viewed from the labial, through the cavity floor. A few small vessels, between eight and ten micra in diameter, are labelled but none were seen away from the cavity.

Original magnification X 50

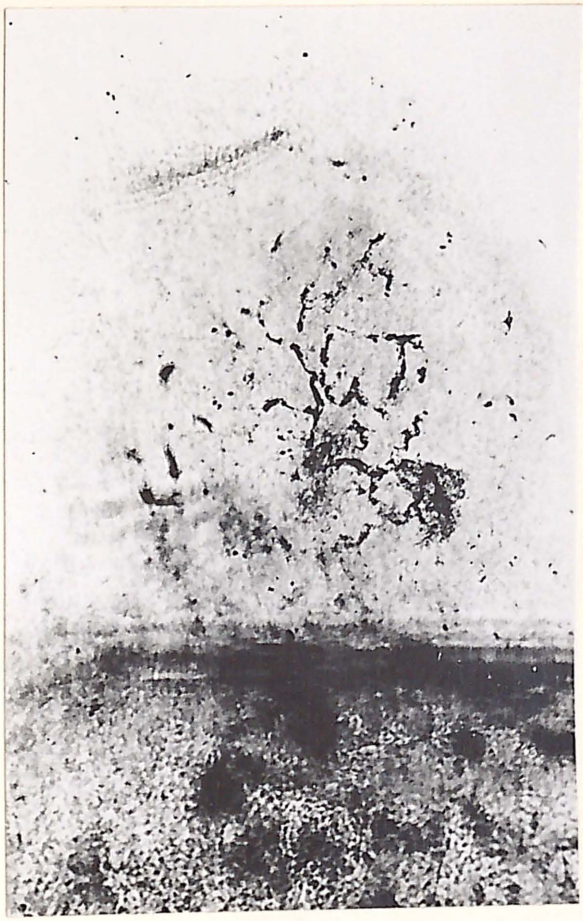
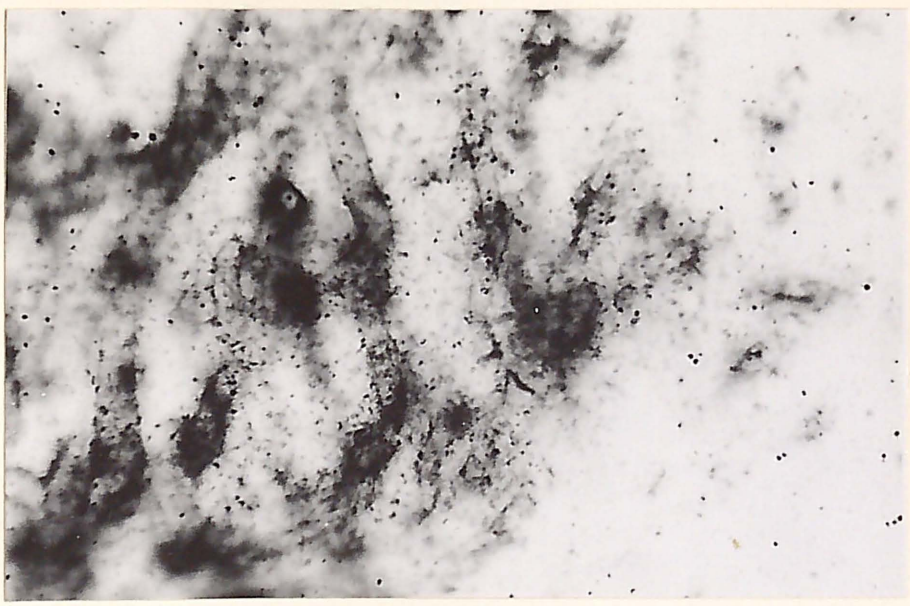


Figure 10. Dog II, tooth +7, a deep cavity with histamine applied. Larger labelled vessels, 40 to 60 micra in diameter, can be seen and are apical to the cavity.

Original magnification X 50



DISCUSSION

A method for studying vascular leakage in inflammation has been adapted for use in investigating the dental pulp. The simple intravenous injection of a suspension of carbon particles, following operative procedures, accurately identifies vessels which have leaked plasma into the surrounding pulp tissue.

The adaptations which have been made from the original study of Majno et al[66] are ; a) decalcification of the teeth prior to clearing and b) the use of methyl salicylate as the clearing solution. It was found that glycerine, used by Majno et al[66] for clearing their tissue, was not effective in clearing dogs' teeth. It was found that to gain the best view of the pulp tissue in the cleared specimens it was desirable to trim the tooth as much as possible with a sharp scalpel, while the teeth were being dehydrated. Methyl salicylate is an excellent solvent, so care must be exercised to avoid damage to equipment and personnel.

Intravascular carbon was found in all the dogs. This was most severe in the third dog but it never obscured the vascular labelling. However in future studies it may well be determined that the animal should be left 24 hours or more after the injection of carbon before sacrifice. The labelling of the vessels lasts indefinitely, carbon which has lodged between the endothelial cell and the basement membrane eventually becomes located in phagocytes lying close to the vessel, labelled vessels are still visible three months after leaking.[31] Leaving the

animal for 24 hours following the administation of carbon would allow time for all of the carbon to be removed from the blood by the reticulo-endothelial system.

A pitfall of the method has been pointed out by Ham and Hurley.[78] They found that in turpentine-induced pleurisy the capillaries (7 to 10 micra in diameter) were often labelled. However, electron micrographs showed that the carbon lay in the lumen of the vessels and indicated thrombus formation following damage to the endothelial cells and not vascular leakage. In the present study it was found that capillaries immediately inder the cavities were often labelled. The histologic sections taken from similar teeth frequently showed small vessels under the cavity with their lumen filled with carbon. The larger vessels only showed carbon in their walls and in the cleared specimens the labelling of these larger vessels was not so intense as that of the smaller ones. Thus it may be that the labelling of the small vessels in this study is solely an indication of intravascular thrombus formation. This study would have been helped if it had been possible to examine some of the pulps under the electron microscope. It is not possible to accurately locate the position of the carbon particles in ordinary histologic sections.

The use of electron microscopy in pathology is still in its early stages but it has already brought to light many new facts in the study of inflammation. The author feels that this instrument has great potential in the field of experimental pathology and its use in the study of pulpal inflammation would be most enlightening.

The histologic sections showed margination of polymorphonuclear leukocytes in the larger vessels beneath the cavity preparations. The significance of this is not known since margination of these cells was also seen in teeth which had not been treated in any way. Margination was not seen in vessels below 15 micra in diameter. It was felt that margination of leukocytes was not a significant indicator of pulpal inflammation.

Emigration of leukocytes was rarely seen. Hurley and Spector[77] have found that there is little emigration of leukocytes into injured tissue until about four hours after trauma. In the present study only a few teeth were left four hours of more between cavity preparation and sacrifice. In these there was a marked accumulation of polymorphonuclear leukocytes in the areas immediately below the cavities. All these teeth were in the group that received histamine application. It is not likely that this is significant but it points out the importance of controlling the time element when comparing the effect of two treatments in a short term study such as this. The cavities on one side of the mouth were left untreated while those on the other side received an application of histamine. It was not noted until later that the cavities which received the histamine were those which had been prepared first. This would be another indication for delaying sacrifice of the dog for a longer period as this would tend to reduce any differences due to time.

An interesting finding was that the small vessels were more noticeable in the odontoblastic layer below the cavity than

elsewhere. This would support the contention of Seltzer and Bender[50] that there are vessels in the odontoblastic layer which only becomes noticeable when the area becomes inflamed.

It has been shown that vascular labelling, especially of larger vessels, indicates vascular leakage.[65,70,73,74,78] This study showed that leaking vessels were confined to the area immediately below the cavity except when histamine was applied to the cavity, when it was found that the labelling extended apically and coronally to the cavity. Also labelling of vessels was not noticeable unless the cavity was deep, i.e. within 50 micra of the pulp.

In the teeth with deep cavities it was found that about 15 minutes after preparation a considerable quantity of fluid had welled up into the cavity. This was assumed to be the same as the "dental-pulp fluid" of Haldi[60] and the "dental lymph" of Fish.[49] Since abnormal leakage was indicated by the fact that the vessels under the cavity were labelled with carbon, doubt could be cast on the assumption of Haldi[62] that the dental-pulp fluid is a normal capillary filtrate. His method of obtaining the pulp fluid was more traumatic than the procedures used in this study, since he made actual exposures into the pulp. In favour of Haldi's suggestion is the fact that the protein concentration in the dental-pulp fluid of the dog is 1.2 percent against a concentration of 6.6 percent ~~of 6.6 percent~~ in the plasma. The protein concentration in an inflammatory exudate is usually 4 to 5 percent.[58] However, the labelling of the vessels in this study would suggest the possibility that the dental-pulp fluid is a capillary exudate.

In Hassan's study it was found that if an intravenous injection of Evans blue was given the dye could be seen in the dental-pulp fluid. Evans blue is usually used to indicate an abnormal permeability of vessels[99] but it does escape normally in small quantities from the smaller venules.[59] Thus the author would like to raise the doubt as to whether the dental-pulp fluid is a capillary filtrate or a capillary exudate.

The study was designed to determine if there was any difference in the leakage in the skin as opposed to the dental pulp. It was found that a considerably larger number of vessels in the skin were labelled than in the dental pulp. It is difficult, however, to draw a valid conclusion from this finding. It could just be due to the fact that more histamine found its way to the skin than into the dental pulp. However, it seems that since there is much more possibility for the tissues of the skin to swell than the tissue of the dental pulp, there might be more loss of fluid from the vessels of the skin. It is probable that the amount of leakage could be assessed more accurately if a protein conjugated to a fluorescent dye were used as a tracer. The author started to use this method but did not pursue the line of approach beyond the unsuccessful preliminary trial.

It would be interesting to determine if the same amount of leakage could be induced without having to cut a cavity so near to the pulp. It was obvious that this allowed a large amount of fluid to escape through the dentinal tubules. If there had not been this line of escape for the fluid it is possible that there would be very little leakage from the pulpal vessels into the

surrounding tissue. The use of heat would be an excellent method for causing the necessary damage without exposing the dentinal tubules. The adapted soldering iron proved to be inadequate for the purpose but a thermoelectric heating device could prove to be useful.

SUMMARY AND CONCLUSIONS

It was found from a perusal of the literature that relatively few had studied the vascular changes in inflammation of the dental pulp. The reaction of the microcirculation to injury is considered to be of the utmost importance in protecting tissues from injury. It was thought that an investigation into the reactions of the vessels of the dental pulp to injury would be of value.

The review of literature includes a brief summary of current concepts of inflammation. The fine structure of small vessels was reviewed in order to provide a basis for the discussion of vascular permeability which followed. A review of the method of "vascular labelling" was included since this was the method used in the present study. The method involves the administration of an intravenous injection of carbon particles which become localized in the walls of vessels which are leaking plasma. The endothelial cells in leaking vessels become partially disconnected along their borders in order to allow the plasma to escape. Carbon particles in the blood get swept into these openings but are trapped in the vessel wall by the filter-like action of the basement membrane, while the plasma passes on into the surrounding tissues. The circulating carbon is rapidly removed by the reticulo-endothelial system so that the only blackened vessels which remain are those which have been leaking plasma.

The present study involved the use of three mongrel dogs and a total of 68 teeth in which cavities of varying depth were prepared. Some of these received applications of histamine phosphate solution. Small quantities of histamine were also injected intradermally in the abdominal wall. Intravenous injections of a dialysed carbon suspension were made and the animal sacrificed one to three hours later. The material from the teeth and the skin was examined both in ordinary histologic sections and as whole cleared tissue. Some components of the reticulo-endothelial system removed from one animal were examined. The carbon particles were concentrated in the spleen and liver.

Labelling of vessels in the dental pulp was not noticeable unless the cavity floor was 50 micra or less in thickness. In this study it was felt that this could well be due to lack of sufficient damage to the pulps of the teeth with shallow cavities. However, it was noted that associated with the preparation of deep cavities there appeared after about 15 minutes,a layer of fluid on the cavity floor. This fluid was assumed to be the dental-pulp fluid and it was suggested that if there had not been this route of escape for the plasma there might not have been much leakage from the pulpal vessels and so , less labelling. It would be desirable to repeat this study using a method of causing injury which did not involve exposing the dentinal tubules. Controlled heat would be an excellent tool.

A comparison of the pattern of leakage in the skin and in the dental pulp showed that there was considerably more leakage in the skin. This finding was thought to be valid but could have

been due to the difficulty in controlling the amount of histamine placed in contact with the pulp compared with the amount injected into the skin. Nevertheless, the results in the two tissues were very similar even if quantitative detail varied. It was concluded that it would be difficult to carry out a study comparing the reactions of the skin and the dental pulp to injury since it is virtually impossible to cause comparable damage to the two tissues. Thus, it would be difficult to carry out the original intent of this study, which was to perform such a comparison.

It was found that labelling of vessels was confined to the area immediately below the cavity preparation. However, when histamine had been applied the labelled vessels could be seen apically and coronally to the area of the cavity. It was thus thought that histamine could diffuse across a small thickness of dentine and had a similar effect on the vessels of the pulp as on those in other parts of the body.

It was considered that the blackening of the smallest vessels, which was very intense, could have been due to the formation of intravascular thrombi and not vascular leakage.

This investigation showed that the method of "vascular labelling" could be adapted to study the dental pulp. While the information gleaned was limited by the size of the sample and technical limitations it was thought that this was a useful and simple method which held promise for further study.

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1953 - 1958	The Leys School, Cambridge, England.
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Professional Societies

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ABSTRACT

A method for studying the permeability of the blood vessels of the dental pulp during acute inflammation

by

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A study was made to develop a method for investigating the quantity and location of vascular leakage during acute inflammation of the dental pulp. Use was made of the method of "vascular labelling" by which carbon particles placed in the blood are deposited in the walls of leaking vessels. Three dogs were used, with cavities of varying depth being cut in their permanent teeth. Vascular leakage was induced in the skin and in some of the teeth by applying histamine phosphate solution. The tissues were studied in normal histologic sections and as cleared specimens. It was found that the number of leaking vessels was much less in the dental pulp than in the skin and that leakage was confined to the area immediately below the cavity preparation unless histamine had been applied to the cavity, when the labelled vessels were found further apically. The number of leaking vessels depended on the depth of the cavity, becoming greater as the cavity depth increased. These findings would support the common finding that the degree of inflammation beneath cavity preparations increases with cavity depth. The conclusion was that the method developed held promise as a tool in investigating vascular changes in the dental pulp.